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Onset of Thymic Recovery and Plateau of Thymic Output Are Differentially Regulated after Stem Cell Transplantation in Children

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ABSTRACT

Thymus-dependent T-cell regeneration is a major pathway for immune reconstitution after stem cell transplantation in children. Therefore, we prospectively assessed T-cell dynamics and thymic function in 164 pediatric patients between 1 and 124 months after transplantation by measuring T-cell receptor recombination excision circles and spontaneous expression of Ki67 in peripheral T-cell subsets. We analyzed the effect of recipient age, conditioning regimen, type of donor and graft, stem cell dose, and graft-versus-host disease on the onset and the plateau of thymic output. A high rate of spontaneous proliferation in early-reconstituting naive and memory T cells inversely correlated with total T-cell numbers. Accordingly, T-cell receptor recombination excision circle content was diminished in early-appearing naive T cells. A multivariate analysis revealed that the onset of thymic recovery was inversely correlated only with recipient age ($P < .0002$), whereas the plateau of thymic output was higher in patients receiving increased stem cell numbers ($P < .0022$). Donor type, stem cell source, and conditioning regimen influenced none of the analyzed parameters. In conclusion, lymphopenia-driven proliferation is important for T-cell homeostasis in children early after stem cell transplantation, but it might result in underestimation of thymic function. Onset and plateau of thymic activity are independently regulated by different transplant-related factors.

KEY WORDS

Thymic function • Immune reconstitution • T-cell dynamics • TREC • Pediatric stem cell transplantation

INTRODUCTION

T-cell reconstitution in lymphopenic hosts has been shown to follow 2 major pathways: peripheral, antigen-driven expansion of activated T cells or de novo generation of naive T cells from hematopoietic stem cells in the host thymus [1,2]. Whereas the first pathway leads to a restoration of memory-type T cells in which diversity of the T-cell receptor (TCR) repertoire is dependent on the number of T-cell precursors in the graft, the latter bears a closer resemblance to normal T-cell ontogeny and results in reconstitution of a fully mature T-cell compartment. In humans, production of naive T cells via the second pathway is dependent on a functional thymus, because in congenitally athymic patients, such as patients with complete DiGeorge syndrome or patients thymectomized before an allogeneic transplantation, no naive T cells are detectable after transplantation [3,4]. However, transplantation of thymic tissue has been able to restore naive T-cell production in 2 DiGeorge patients [5]. Until recently, it has been difficult to quantify thymic function in humans precisely because of the lack of specific markers on recent thymic emigrants (RTEs) and the uncertainty of ascribing T cells with a naive phenotype to those recently produced in the thymus. Novel insights into the mechanisms operational during TCR rearrangement [6,7] have led to the intro-

duction of the TCR excision circle (TREC) assay [4,8], which has been used to quantify thymic function in a variety of clinical situations [9-17]. However, interpretation of TREC data is complex, because the TREC content in the peripheral T-cell compartment is influenced not only by thymic output, but also by longevity of naive T cells and by dilution of TRECs through ongoing T-cell divisions. It has therefore been suggested that TREC data can be interpreted only together with information about the actual T-cell turnover state [18,19].

After stem cell transplantation, successful reconstitution of naive T cells has been shown to restore the diversity of an initially skewed TCR repertoire [11,20], to increase the secretion of interleukin 2 and expression of CD69 on peripheral T cells [21], and to result in a lower risk of disease recurrence after autologous transplantation [16,22]. Because of an increasing awareness of the importance of naive T-cell regeneration, a number of studies have recently analyzed TREC values in the context of immune reconstitution after stem cell transplantation [11,13,14,17,23-27]. There is a general consensus that TREC content in peripheral T cells is low immediately after transplantation and that the thymus contributes substantially to naive T-cell regeneration even in adult patients. Most investigators have found a positive correlation between TREC content and naive T-cell numbers, as well as TCR repertoire diversity [11,17,23]. Most studies were performed in adult patients, and in this age group, a negative correlation was described between the patient's age and TREC values [11,24]. Only 1 study included a small cohort of children, and this group exhibited significantly higher TRECs than the larger adult population [27]. Data on other transplant-related factors, such as conditioning regimen, type of graft, cell dose, and acute or chronic graft-versus-host disease (GVHD), are less clear. Although chronic GVHD and a history of resolved GVHD were associated with low TREC values in 2 studies [17,27], this could not be confirmed by Storek et al. [24]. Regarding the type of graft, higher TRECs were reported for T cell–replete grafts, but only in the first 9 months after transplantation or in cases in which children were excluded from the analysis [27]. Douek et al. [11], reported slightly higher TRECs in recipients of $CD34^+$-selected grafts after 2 years. To date, only 1 study, performed by Hazenberg et al. [13], has taken into account the increased T-cell division rates after transplantation, as measured by the intracellular expression of Ki67. These authors found that TREC content after transplantation was determined by T-cell divisions related to clinical events rather than by homeostatic adaptation to lymphopenia.

In this prospective study, we investigated a pediatric cohort of 164 children who underwent transplantation at the Children's Hospital of the University of Tübingen between 1991 and 2003. Our cohort could be divided into 4 major categories: recipients of stem cells from matched sibling, unrelated, or mismatched-related donors or of autologous stem cells. A multifactorial approach was applied to study the influence of several variables—namely, recipient age, conditioning regimen, stem cell source and dose, type of donor, and acute or chronic GVHD—not only on the plateau of TREC values, but also on the onset of thymic activity. Furthermore, we determined the T-cell turnover state during different stages of immune reconstitution by measuring the spontaneous expression of Ki67 in naive and memory $CD4^+$ and $CD8^+$, as well as effector $CD8^+$, populations. Our results indicate that thymic function may be substantially underestimated when only TREC values or peripheral naive T-cell numbers are used as a surrogate marker for thymic output. In children, homeostatic proliferation seems to be an important regulator of T-cell pool size soon after transplantation and can result in rapid clearance of $TREC^+$ T cells from the periphery. We describe the factors that influence thymic output and the onset of thymic activity, namely, recipient age and transplanted stem cell dose.

PATIENTS AND METHODS

Patients

Between February 2001 and July 2003, a total of 373 samples from 164 children were collected after myeloablative conditioning and stem cell transplantation in the outpatient department of our pediatric stem cell transplant program. Patients had undergone transplantation between November 1991 and April 2003 at the Children's Hospital of the University of Tübingen. Eighty-three patients underwent transplantation after May 2000 (median age, 7.5 years; range, 0.3-22.6 years) and were monitored prospectively to determine the parameters T_{min} and *PL* (plateau level). T_{min} describes the earliest time point when TRECs become detectable in a patient after transplantation, and *P* is a measure of the plateau of thymic output, which is generally achieved after 1 to 2 years. Three samples per patient (median; range, 1-9) were analyzed in this cohort, with a median follow-up of 333 days after transplantation (range, 50-1105 days). Eighty-one patients who underwent transplantation before May 2000 (median age, 7.3 years; range, 0.5-19.4 years) could be analyzed only in the plateau phase (*PL*) of their thymic function. Because in this phase TREC values are considered to be relatively stable (see Statistical Analysis), only 1 sample per patient (median; range, 1-3) was analyzed in this subgroup, with a median follow-up duration of 1507 days after transplantation (range, 320-3715 days) to estimate *PL*. The study was approved by the institutional review board, and parental consent was obtained according to institutional guidelines.

Patients not surviving beyond day 100 were excluded, whereas patients successfully grafted after a second transplantation that was necessary because of graft rejection or nonengraftment or tandem high-dose chemotherapy with autologous stem cell rescue were included in the study. The cohort could be grouped into 4 major categories: recipients of autologous stem cells (n = 51) or of cells from an HLA-matched sibling (n = 47), unrelated (n = 45), or parental (haploidentical) donor (n = 21). From the 110 patients receiving peripheral blood stem cells (PBSCs), only 6 received unmanipulated PBSCs; all remaining 104 patients received highly purified, $CD34^+$-selected PBSCs (resulting in a 3-4 log T-cell depletion) without any posttransplantation immunosuppressive therapy. GVHD prophylaxis for recipients of unmanipulated grafts consisted of cyclosporin A for the first 100 to 200 days with subsequent tapering. Conditioning regimens were based either on busulfan or total body irradiation for allogeneic transplantations or on melphalan/carboplatin for autologous transplantations. Patient characteristics are listed in Table 1. The primary indication for transplantation was a malignant disorder such as leukemia or lymphoma (n = 62) or solid tumor (n = 45). Thirty-six patients underwent transplantation, however, because of a nonmalignant disease (Table 2).

Table 1. *Patient Characteristics (n = 164)*

Characteristic	n
Autologous transplantations (n = 51)	
Median age, 4.8 y (range, 1-19.4 y)	
Transplantation date, November 1991 to January 2003	
Follow-up duration, median, 712 d (range, 50-3715 d)	
Solid tumor	42
Leukemia/lymphoma	7
Nonmalignant disease (scleroderma, Morbus Still)	2
Peripheral blood stem cells ($>10^7/<10^7$ $CD34^+$/kg BW)	48 (15/33)
Bone marrow	3
Conditioning (TBI based/busulfan based/other)	3/5/43
Matched sibling donor transplantations (n = 47)	
Median age, 8.3 y (range, 0.7-19 y)	
Transplantation date, June 1992 to October 2002	
Follow-up duration, median, 1107 d (range, 99-3703 d)	
Leukemia/lymphoma	22
Solid tumor (rhabdomyosarcoma)	2
Myelodysplastic syndrome	8
Nonmalignant disease	15
Peripheral blood stem cells ($>10^7/<10^7$ $CD34^+$/kg BW)	11 (3/8)
Bone marrow	36
Conditioning (TBI based/busulfan based/other)	18/26/3
GVHD (acute/chronic)	27/6
Unrelated donor transplantations (n = 45)	
Median age, 8.9 y (range, 0.3-22.6 y)	
Transplantation date, February 1993 to April 2003	
Follow-up duration, median, 509 d (range, 76-3065 d)	
Leukemia/lymphoma	30
Myelodysplastic syndrome	3
Nonmalignant disease	12
Peripheral blood stem cells ($>10^7/<10^7$ $CD34^+$/kg BW)	32 (13/19)
Bone marrow	13
Conditioning (TBI based/busulfan based/other)	23/20/2
GVHD (acute/chronic)	10/3
Parental donor (haploidentical) transplantations (n = 21)	
Median age, 7.5 y (range, 0.5-18.3 y)	
Transplantation date, March 1995 to March 2003	
Follow-up duration, median, 735 d (range, 53-2763 d)	
Leukemia/lymphoma	12
Solid tumor	1
Myelodysplastic syndrome	2
Nonmalignant disease	6
Peripheral blood stem cells ($>10^7/<10^7$ $CD34^+$/kg BW)	19 (14/5)
Bone marrow	2
Conditioning (TBI based/busulfan based/other)	13/5/3
GVHD (acute/chronic)	2/3

BW indicates body weight; GVHD, graft-versus-host disease; TBI, total body irradiation.

Cell Preparation

Peripheral blood was collected in ethylenediaminetetraacetic acid–containing tubes, and peripheral mononuclear cells (PMNCs) were subsequently isolated by Ficoll-Hypaque density gradient centrifugation. After 2 washes, cell pellets were frozen immediately at −80°C and stored until analysis of TRECs by quantitative polymerase chain reaction (PCR). The content of $CD3^+$ T cells in the PMNC fraction was determined by flow cytometry on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) by using CD3 PerCP-labeled monoclonal antibodies before freezing. In parallel, the percentages and absolute numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in each sample, as well as the naive and memory populations in each subset (eg, expression of CD45RA/CD45RO on $CD4^+$ and CD45RA/CD27 on $CD8^+$ T cells), were determined in freshly isolated cells by flow cytometry by using standard procedures. All monoclonal antibodies were purchased from Becton Dickinson. Starting in March 2002, the spontaneous intracellular expression of Ki67 was analyzed in parallel in freshly isolated naive and memory $CD4^+$ and $CD8^+$ populations within 12 hours in all samples (in total, 141 samples from 81 patients). In brief, after staining of cell-surface markers and fixation with 4% paraformaldehyde, cells were permeabilized with 0.3% saponin (Riedel deHaen, Deisenhofen, Germany), stained intracellularly with Ki67 fluorescein isothiocyanate (Coulter-Immunotech, Krefeld, Germany), washed twice in 0.1% sa-

Table 2. *Transplantation for Nonmalignant Diseases*

Data	n
Median age, 4.8 y (range, 0.3-19.4 y)	
Transplantation date, December 1992 to January 2003	
Follow-up duration, median, 897 d (range, 88-3613 d)	
Congenital granulocyte disorder	1
Congenital red blood cell defect	2
Hemophagocytic lymphohistiocytosis	1
Metachromatic leukodystrophy	1
X-chromosomal adrenoleukodystrophy	3
Autoimmune diseases	2
Osteopetrosis	5
Polycythemia vera	1
Severe aplastic anemia	11
Severe combined immunodeficiency	1
Shwachman-Diamond syndrome	1
Sickle cell disease	2
Thalassemia	3
Wiskot-Aldrich syndrome	2
Total	36

ponin, and analyzed on a FACSCalibur by using CellQuest software (Becton Dickinson) [28].

Quantitative Real-Time PCR

Quantification of TRECs in isolated PMNCs was performed by real-time quantitative PCR by means of the 5′ nuclease TaqMan assay with an ABI7700 system (PerkinElmer, Weiterstadt, Germany), as previously described [4,11]. Cells were incubated with proteinase K 100 μg/mL (Roche, Mannheim, Germany) at a ratio of 10 μL per 10^5 cells for 1 hour at 56°C and then for 15 minutes at 95°C. Real-time quantitative PCR was performed on 5 μL of cell lysate (equivalent to 50 000 cells) with the primers 5′-CACATCCCTTTCAACCATGCT and 5′-GCCAGCTGCAGGGTTTA-GG and TaqMan probe FAM-5′-ACACCTCTGGTTTTTGTAAAG-GTGCCCACT-TAMRA (MegaBases, Chicago, IL). PCR reactions contained 0.5 U of Platinum Taq polymerase (Invitrogen, Karlsruhe, Germany), 3.5 mmol/L $MgCl_2$, deoxynucleoside triphosphate solution 0.2 mmol/L, 500 nmol/L of each primer, and 200 nmol/L probe in a final volume of 25 μL. Conditions were 95°C for 5 minutes, 95°C for 30 seconds, and 60°C for 1 minute for 40 cycles. A standard curve was plotted, and TREC values for samples were calculated by the ABI7700 sequence-detector software. Samples were analyzed in triplicate.

Magnetically Activated Cell Sorting Separation of Naive and Memory T-Cell Subpopulations

Peripheral blood mononuclear cells were collected from healthy adult controls (n = 5) and patients before (n = 10) and 6 months after transplantation (n = 4) by using standard procedures. In the early posttransplantation phase, when memory T cells dominated the peripheral T-cell pool, peripheral blood mononuclear cells were magnetically activated cell sorted into $CD4^+$ and $CD8^+$ cells by following the manufacturer's protocol, and purity was confirmed by fluorescence-activated cell sorting analysis. When >10% $CD4^+/CD45RA^+$ naive-type T cells were detectable in the peripheral blood, peripheral blood mononuclear cells were sorted into naive ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ and $CD8^+$ cells by using the $CD4^+$-Multisort-Kit (Miltenyi, Bergisch-Gladbach, Germany). The median purity of the target populations was 87.1% (range, 39.7%-99.8%). Subsequently, the TREC content of the respective populations was measured as described previously.

Statistical Analysis

All patients analyzed in the early posttransplantation phase had at least 1 TREC-negative sample. On the basis of this observation and the course of TREC values displayed in Figure 1, we developed the following model of TREC reconstitution: after an early phase without detectable $TREC^+$ T cells in the peripheral blood, patients start to reconstitute $TREC^+$ T cells at a time point T_{min} during the first year after transplantation. Approximately 18 to 24 months after transplantation, a stable plateau *PL* of $TREC^+$ T cells is achieved in which thymic T-cell neogenesis and clearance of $TREC^+$ T cells from the peripheral blood are in balance. The parameter T_{min} was assumed to have a log-normal distribution whose median depends linearly on age, because a preliminary analysis based on regression of T_{min} and age grouped according to quartiles had shown a linear association. Interval-censoring of the observations because of missing values was taken into account (81 patients

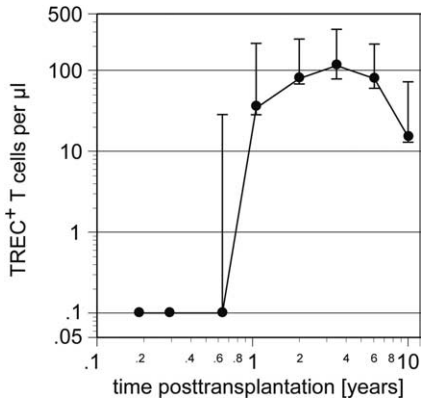

Figure 1. $TREC^+$ peripheral T cells after stem cell transplantation. TRECs were not detectable in the early phase after transplantation but started to increase at time point T_{min}. After approximately 1 to 2 years, $TREC^+$ T cells reached a stable plateau *PL*, which remained constant for up to 10 years after transplantation. TREC values from all patients were plotted against time after transplantation. Results are shown as median values; error bars represent 25th and 75th percentiles.

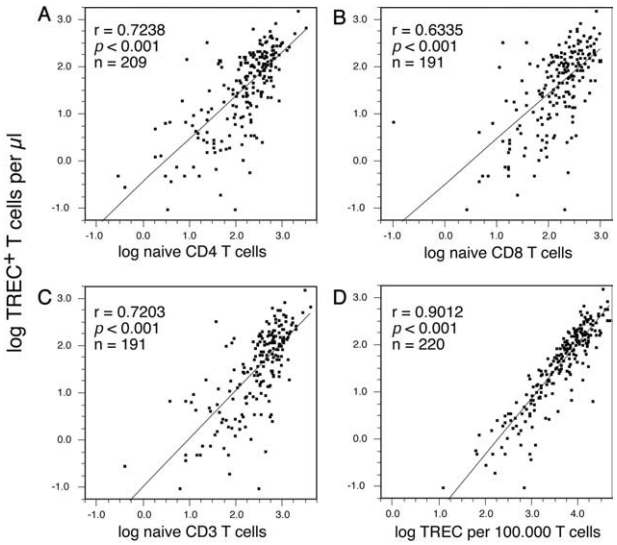

Figure 2. Correlation of TRECs with naive T-cell populations. After logarithmic transformation, TREC levels correlated with the numbers of naive $CD4^+$ (A) and naive $CD8^+$ (B) cells and with the total number of naive $CD3^+$ T cells (C). Furthermore, TREC content, given as TRECs per 100 000 T cells, correlated well with the calculated number of $TREC^+$ T cells per microliter (D). This indicates that both parameters may be used equivalently and that correction for peripheral T-cell numbers is usually not necessary; n indicates the number of analyzed samples.

were studied only in the plateau phase *PL*). The parameters were estimated by maximum likelihood. The effect of the following variables on T_{min} of $TREC^+$ T cells was analyzed: type of transplantation (autologous, matched sibling, unrelated, or parental donor), type of disease (malignant or nonmalignant), source of stem cells (PBSCs or bone marrow), transplanted $CD34^+$ cell number ($\geq 10^7$ or $<10^7$ $CD34^+$ cells per kilogram body weight), conditioning regimen (total body irradiation based, busulfan based, or other), and presence of GVHD (acute or chronic). Because only age and acute GVHD showed a significant effect on T_{min}, these 2 factors were entered simultaneously into a multifactorial maximum-likelihood model. The plateau level *PL* for the 101 patients who achieved TREC-positive values was estimated by their geometric means and compared for the various subgroups by 1-way analysis of variance. To achieve equal variances among groups, a logarithmic transformation was applied. A stepwise multifactorial analysis of variance was performed that initially entered all the factors mentioned previously for $T_{min.}$. A *P* value of .05 was set as the selection criterion.

Assuming a detection limit of Ki67 of 0.1%, we compared Ki67 expression before and after day 180 by using restricted maximum likelihood by taking the censored observations below the detection limit explicitly into account. The logit was defined as the logarithm of the odds, ie, the fraction divided by 1 minus the fraction. The different number of samples among patients was taken into account by considering patient number as a random factor. We correlated the expression of Ki67 in 5 T-cell subpopulations with each other and with the numbers of peripheral $CD3^+$ T cells. The *P* values were adjusted according to the Bonferroni-Holm method for the number of hypotheses tested in the same data set. The same statistical method was used to compare the TREC content in magnetically activated cell-sorted T-cell subpopulations. All pairs of T-cell subsets were compared by using the Tukey-Kramer honestly significant different test with $\alpha = .05$.

RESULTS

TREC Content, Absolute TRECs, and Naive T-Cell Numbers

TREC values were calculated as absolute numbers of $TREC^+$ T cells per microliter of peripheral blood and as $TREC^+$ T cells per 100 000 T cells. Values of $TREC^+$ T cells per microliter showed a strong positive correlation with the numbers of naive $CD4^+$ T cells (Figure 2A) and naive $CD8^+$ T cells (Figure 2B), as well as with the total number of naive $CD3^+$ T cells (Figure 2C). Similar results were obtained for the correlation between $TREC^+$ T cells per 100 000 T cells and naive T-cell numbers (data not shown). Therefore, under normal circumstances, naive T-cell numbers in the peripheral blood may be used to estimate TREC values. Furthermore, there was a strong positive correlation between the number of $TREC^+$ T cells per microliter and $TREC^+$ T cells per 100 000 T cells (Figure 2D), thus indicating that both parameters can be used equivalently. Data are presented as $TREC^+$ T cells per microliter, because this parameter is a better indicator of thymopoiesis. Overall numbers of peripheral $TREC^+$ T cells after stem cell transplantation are shown in Figure 1.

Intracellular Expression of Ki67 in Peripheral T Cells

Relative representation of the 4 major categories (matched related, unrelated, haploidentical, and autologous transplants) in the 81 patients in whom Ki67 expression was studied was not different from that in the total population (data not shown). Spontaneous expression of Ki67 in peripheral T cells was significantly higher in patients studied before day 180 (mean, 1.8%; 95% confidence interval [CI], 1.33%-2.54%; n = 27) than after day 180 after transplantation (mean, 0.83%; 95% CI, 0.68%-1.03%; n = 65). Ki67 expression was positively correlated between all T-cell subsets (naive CD4/memory CD4, naive CD4/naive CD8, naive CD4/memory CD8, memory CD4/naive CD8, memory CD4/memory CD8, and

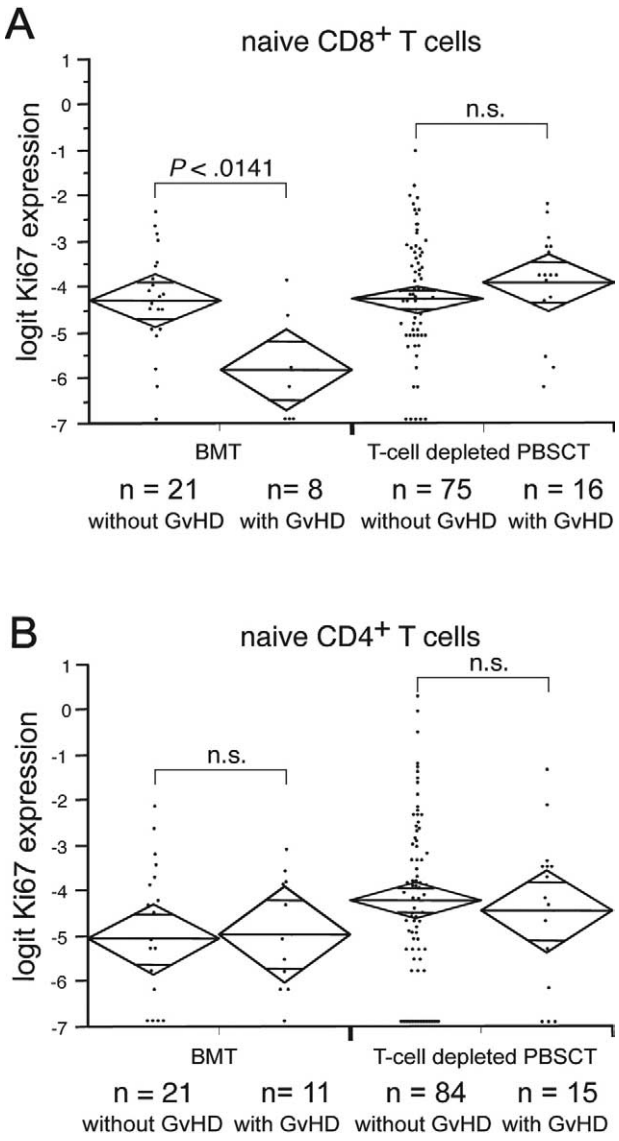

Figure 3. Acute GVHD reduces Ki67 expression in naive $CD8^+$ T cells. Acute GVHD (grade II-IV) resulted in a reduced expression of Ki67 in naive $CD8^+$ T cells after transplantation of unmanipulated bone marrow from matched sibling donors. However, this did not reach statistical significance after transplantation of T cell–depleted peripheral blood stem cells (A). No effect of acute or chronic GVHD on Ki67 expression in naive $CD4^+$ T cells (B) or in memory $CD4^+$, memory $CD8^+$, and cytotoxic T lymphocyte $CD8^+$ populations could be observed (data not shown). Results were compared by 1-way analysis of variance. Diamonds represent means ± 95% confidence intervals; n indicates the number of analyzed samples.

naive CD8/memory CD8; correlation coefficient, 0.29-0.46), and this indicates that proliferation in these populations is likely to be driven by a common factor. It is interesting to note that Ki67 expression in naive and memory $CD4^+$ and $CD8^+$ T cells was inversely correlated with $CD3^+$ T-cell numbers, thus suggesting that spontaneous proliferation of these populations in the early posttransplantation phase is driven by homeostatic mechanisms (correlation coefficient, −0.28 to −0.38). Furthermore, we were unable to detect a significant influence of T-cell depletion or GVHD on Ki67 expression in most populations. However, naive $CD8^+$ T cells, but not naive $CD4^+$ T cells, of patients with a history of GVHD after unmanipulated bone marrow transplantation showed a significantly lower expression of Ki67 (mean, 0.3%; 95% CI, 0.8%-2.3%; and mean, 1.4%; 95% CI, 0.1%-0.7% in patients with versus without GVHD, respectively; $P = .0141$). This phenomenon could not be found in patients with GVHD after $CD34^+$-selected PBSCs (Figure 3). In contrast, Ki67 expression in effector $CD8^+$ T cells was not correlated with any of the analyzed parameters. Therefore, the proliferation of effector $CD8^+$ T cells seems to be driven by other factors that were not evaluated in this study.

Factors Influencing the Onset of Thymic Activity

The onset of thymic activity was defined as the time point T_{min}, when patients switched from $TREC^-$ to $TREC^+$ samples. In the 83 patients in whom T_{min} could be studied, relative representation of the 4 major categories (matched related, unrelated, haploidentical, and autologous transplants) was not different from that of the total study population (n = 164). In the first step of analysis, we split our cohort of patients into 4 quartiles according to age. From this model, we calculated that the median time to switch is 83 days for children younger than 1 year at transplantation and increases by 14 days with every year of life. In addition, there was a positive correlation between patient age and T_{min} (Figure 4). The median time up to the switch to positive TREC values occurred almost 2 months earlier in patients who underwent transplan-

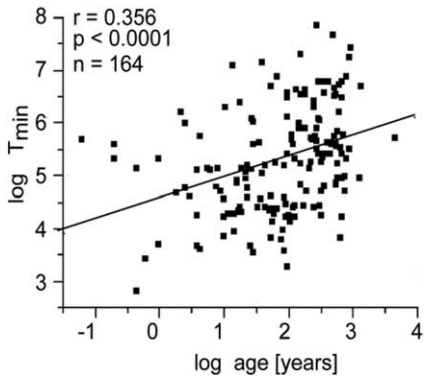

Figure 4. The onset of thymic function T_{min} is positively correlated with patient age. The onset of thymic function T_{min} was estimated by using a maximum-likelihood model, explicitly taking into account missing values by interval-censoring the observations. A preliminary analysis based on regression of T_{min} and age grouped according to quartiles showed a linear association. In a model of linear fit, T_{min} was positively correlated with patient age ($P < .0001$).

tation on account of a nonmalignant disease compared with patients who had malignant diseases (median: 206 versus 148 days in patients with malignant versus nonmalignant diseases, respectively). However, because of the unequal distribution of samples in the 2 groups (n = 128 versus n = 36), this difference failed to reach statistical significance ($P = .21$; Table 3). Type of graft, donor, conditioning regimen, and transplanted stem cell number had no effect on the onset of thymic activity. Furthermore, there was no statistically significant difference for T_{min} between autologous and allogeneic transplantations. In contrast to the beneficial effects of younger age and nonmalignant disease, acute GVHD (grade II-IV) and chronic GVHD (limited and extensive) delayed the switch to TREC positivity by 151 days (median; $P = .036$) and 184 days (median; $P = .16$), respectively. However, subjecting age and acute GVHD to a multifactorial model, only age could be confirmed to have a significant effect on T_{min}. Although acute GVHD delayed the onset of thymic recovery by 149 days (mean; 95% CI, −2 to 378 days), this difference was not significant because of the inclusion of 0 in the 95% CI.

To rule out alternative explantations for the association between patient age and T_{min}, we examined whether an earlier arrest of peripheral expansion in younger children might lead to higher TREC levels. However, we could not detect any correlation between patient age and Ki67 expression, either in naive and memory $CD4^+$ and $CD8^+$ T cells or in $CD8^+$ cytotoxic T lymphocytes (data not shown). Furthermore, the distribution of T cell–depleted versus T cell–replete grafts was not different among age quartiles (30 T cell–depleted versus 10 T cell–replete grafts in patients <3.7 years; 30 versus 12 in patients between 3.8 and 7.3 years; 25 versus 16 in patients between 7.4 and 12.4 years; and 31 versus 10 in patients >12.6 years; $P = .43$). Thus, the positive correlation between patient age and T_{min} cannot be explained by differences in homeostatic proliferation or graft composition.

Factors Influencing the Plateau Level of TREC Values

After the initial increase in TREC values, patients achieved a plateau level *PL* of TRECs after 18 to 24 months that remained constant over time (Figure 1). We considered *PL* in this case as a second parameter that describes thymic function in a more stable phase and analyzed the influence of the above-mentioned variables on the height of *PL*. It is interesting to note that the only variable that had a significantly positive effect on *PL* was transplanted stem cell number. Patients who received more than 10^7 $CD34^+$ PBSCs per kilogram body weight had significantly higher TRECs in the plateau phase than patients who received lower stem cell doses (8878 versus 3268 median TRECs per 100 000 T cells in patients with higher versus lower stem cell numbers; $P = .002$; Figure 5A). However, there was no positive correlation between stem cell dose and the plateau of thymic function (Figure 5B), thus suggesting that this phenomenon is a threshold effect rather than a linear association. All other analyzed factors, including age and type of disease, failed to have a beneficial effect on thymic output. Furthermore, *PL* did not differ between autologous or allogeneic transplantations. As in the analysis of the onset of thymic activity, in a 1-way analysis of variance, both acute (grade II-IV) and chronic (limited and extensive) GVHD significantly impaired thymic function and resulted in lower TREC levels in the plateau phase (median TRECs per 100 000 T cells: 2707 versus 5102 in patients with versus without acute GVHD [$P = .0395$] and 1381 versus 4628 in patients with versus without chronic GVHD [$P = .0359$]; Figure 5C and D). However, in a multivariate analysis, cell dose (>10^7 $CD34^+$ PBSCs per kilogram) remained the only parameter associated with higher TREC levels in the plateau phase *PL* of thymic output ($P = .0022$; Table 3).

In 4 patients analyzed more than 10 years after transplantation, there seemed to be a substantial decline in TREC values (Figure 1). Therefore, we repeated our analysis after exclusion of these patients. Even with this less inclusive definition, $CD34^+$ cell dose remained the only parameter with a significant effect on the plateau of thymic function in a multivariate analysis ($P = .002$).

TREC Values in Sorted Naive and Memory T-Cell Subpopulations

As expected, in healthy adult controls, TREC levels were high in purified naive $CD4^+$ and $CD8^+$ T cells but remained low or even undetectable in the corresponding memory populations (Figure 6). On the basis of the assumption that naive T cells in the peripheral blood mirror the thymic output of RTEs, one would expect to find a high TREC content in naive T cells repopulating the empty T-cell pool after transplantation. However, TREC content in sorted naive $CD4^+$ and $CD8^+$ T cells in the first 6 months after transplantation was substantially diminished (Figure 6), thus indicating that TRECs in this phase of immune reconstitution are rapidly cleared in the periphery by an increased rate of cell division or by apoptotic cell death of RTEs. After 6 months, when Ki67 expression had significantly declined (described previously), TREC content in naive $CD4^+$ and $CD8^+$ T cells normalized rapidly but remained low or undetectable in memory T cells (Figure 6).

Table 3. *Factors Influencing Onset T_{min} and Plateau Level P of Thymic Activity*

Variable	T_{min} at Median Age of 7.4 y (d)	*P* Value	Plateau TRECs (TRECs per 10^5 T cells)	*P* Value
Age quartiles (≤3.7|3.8-7.3|7.4-12.4|≥12.6| y)	109 versus 166 versus 216 versus 298	<.002 (n = 40 versus 42 versus 41 versus 41)*	5378 versus 5765 versus 3139 versus 3047	.21 (n = 30 versus 27 versus 23 versus 21)
Cell dose (<10^7 versus >10^7 $CD34^+$/kg BW)	190 versus 179	.65 (n = 71 versus 43)	3268 versus 8878	.002 (n = 37 versus 29)†
Graft (BM versus TCD PBSC)	162 versus 199	.46 (n = 54 versus 104)	3239 versus 5107	.25 (n = 39 versus 59)
Donor (matched sibling versus unrelated versus parental versus autologous)	187 versus 195 versus 187 versus 187	.84 (n = 35 versus 45 versus 18 versus 51)	3050 versus 3002 versus 7388 versus 6599	.053 (n = 35 versus 22 versus 11 versus 33)
Conditioning (TBI versus busulfan versus other)	164 versus 191 versus 211	.58 (n = 57 versus 55 versus 50)	3056 versus 3785 versus 6812	.058 (n = 29 versus 39 versus 32)
Acute GVHD (no acute GVHD versus acute GVHD grade II–IV)	164 versus 313	.036 (n = 121 versus 36)*	5102 versus 2707	.0395 (n = 74 versus 27)†
Chronic GVHD (no chronic GVHD versus chronic GVHD [limited & extensive])	182 versus 366	.16 (n = 152 versus 12)	4628 versus 1381	.0359 (n = 95 versus 6)†
Disease (malignant versus nonmalignant)	206 versus 148	.21 (n = 128 versus 36)	3996 versus 5235	.38 (n = 73 versus 28)

Influence of variables on T_{min} was estimated by maximum likelihood, and significance was calculated with the likelihood ratio test. Age dependency of T_{min} could be confirmed for all variables; results for T_{min} are given at the median age of the cohort (7.4 years). Plateaus of TRECs were compared by 1-way analysis of variance; n indicates the number of samples analyzed in each group.

BW indicates body weight; TCD PBSC, T cell–depleted peripheral blood stem cells; TBI, total body irradiation.

*Entering age and acute GVHD in a multifactorial model for estimation of T_{min}, only younger recipient age resulted in a shorter time to T_{min}. The influence of acute GVHD did not remain significant (delay of T_{min} by acute GVHD: 149 days; 95% CI, −2 to 378 days).

†In a multivariate analysis, only cell dose was associated with higher plateau TREC levels ($P = .0022$).

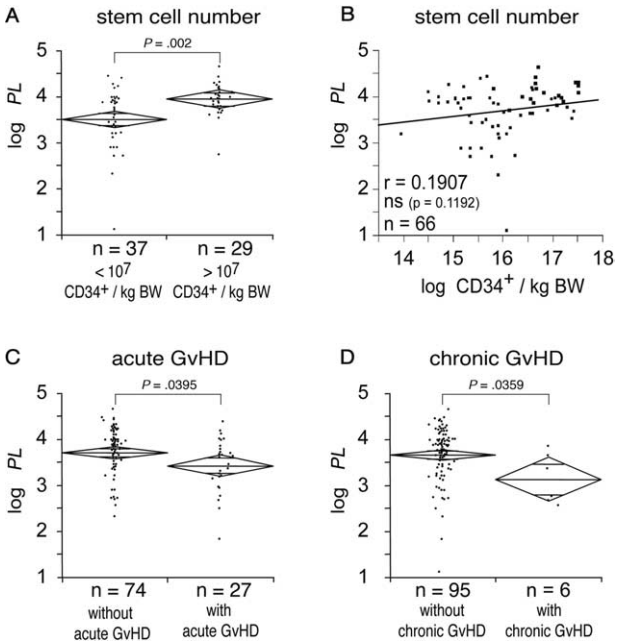

Figure 5. Influence of transplanted stem cell number on the plateau level *PL* of TRECs. Patients receiving more than 10^7 $CD34^+$ cells per kilogram body weight had significantly higher TRECs in the plateau phase of thymic reconstitution (A). However, the association between stem cell dose and plateau level *PL* was not linear (B), thus suggesting that 10^7 $CD34^+$ cells per kilogram body weight is a threshold dose for a better thymic output. Younger age and nonmalignant disease, which resulted in a faster onset of thymic recovery, did not influence TREC levels in this phase. In a 1-way analysis of variance, both acute GVHD (C) and chronic GVHD (D) decreased TREC levels significantly, whereas other transplant-related factors, such as conditioning regimen and type of donor or graft, had no effect (data not shown). However, a multivariate analysis confirmed only stem cell number to have a significant effect on *PL*. Diamonds represent means ± 95% confidence intervals; n indicates the number of analyzed samples.

DISCUSSION

Recently, accumulating evidence for the persisting activity of the thymus in adults and its importance for naive T-cell regeneration after stem cell transplantation or human immunodeficiency virus infection has led to a number of studies focusing on thymic function in such clinical situations. Although the thymus is considered to be most active before the onset of puberty, data on thymic function based on the assessment of TREC levels in children after stem cell transplantation have been scarce. Only 1 study included a small cohort of children among a larger group of adults [27]; a few studies dealt with TREC reconstitution in children with immunodeficiencies [5,26]. Our aim in this study was therefore to analyze T-cell dynamics and thymic function in a purely pediatric cohort of patients after autologous and allogeneic stem cell transplantation. Although the TREC assay represented a very important tool for immune monitoring [4,8], it soon became clear that the interpretation of TREC data can be hampered by the rapid clearance of $TREC^+$ T cells from the peripheral blood or by the longevity of resting $TREC^+$ naive T cells [18,19]. In situations in which no steady-state conditions for the peripheral T-cell pool can be assumed, information about the T-cell turnover state must therefore be taken into account to interpret TREC data correctly. By combining the quantification of TRECs in PMNCs and sorted T-cell populations with flow cytometry of peripheral T-cell subsets and simultaneous measurement of intracellular Ki67 expression, we were able to identify novel aspects of T-cell dynamics after stem cell transplantation in children.

The early period of immune reconstitution after stem cell transplantation is characterized by a high rate of spontaneous T-cell divisions, as determined by the intracellular expression of Ki67 [13]. Ki67 is a nuclear antigen that is expressed in all phases of the cell cycle except G_0, and its expression correlates with T-cell proliferation measured by thymidine incorporation assays [29]. Expression of Ki67 was not found to differ significantly between naive and memory $CD4^+$ and $CD8^+$ T-cell populations but was correlated among all subsets; this indicates that there is a com-

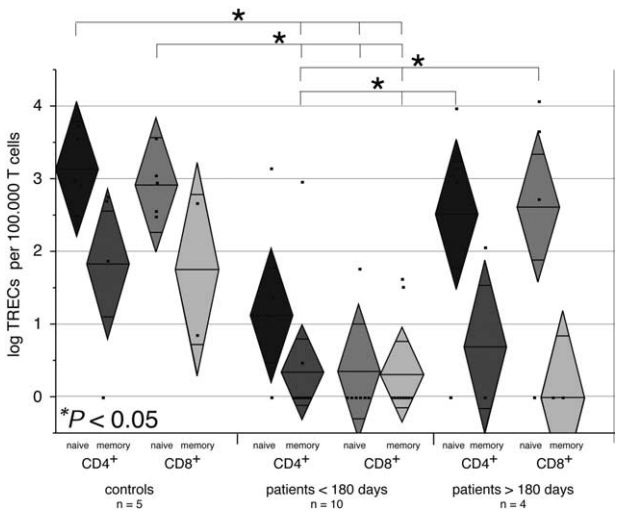

Figure 6. TREC content in sorted naive and memory $CD4^+$ and $CD8^+$ T cells. TRECs were high in sorted naive $CD4^+$ and $CD8^+$ T cells from healthy adult controls but were very low in the corresponding memory populations. In transplant recipients during the first 180 days after transplantation, TREC content in naive T cells was substantially diminished, thus indicating rapid clearance of $TREC^+$ T cells from the naive T-cell pool. After 6 months, when Ki67 expression had significantly declined, TREC content in the naive $CD4^+$ and $CD8^+$ T-cell populations of the patients rapidly normalized, whereas TRECs remained undetectable in memory T cells. Logarithmic transformation of TRECs was applied to achieve equal variances among groups. Diamonds represent means ± 95% confidence intervals; n indicates the number of analyzed samples. Comparisons for all pairs were made with the Tukey-Kramer test.

mon factor that drives T-cell expansion soon after transplantation and that affects all T-cell populations in a similar fashion. The only parameter that was inversely correlated with Ki67 expression in all T-cell subsets except effector $CD8^+$ T cells was the number of peripheral $CD3^+$ T cells. This finding strongly favors the concept that lymphopenia itself is a major driving force for homeostatic proliferation of T cells in the context of immune reconstitution after stem cell transplantation [30,31]. We could not confirm the results of Hazenberg et al. [13], who identified only GVHD and infection as factors responsible for T-cell proliferation but did not detect a correlation between Ki67 expression and peripheral T-cell numbers. In contrast, in naive $CD8^+$ T cells from children with acute GVHD after bone marrow transplantation, we found significantly reduced expression of Ki67. In $CD4^+$ and $CD8^+$ memory and $CD8^+$ effector T-cell populations, there was also a trend toward a lower expression of Ki67 in patients with GVHD, although this did not reach statistical significance (data not shown). This discrepancy might be explained by the different ages of the study populations and by the fact that different stages of GVHD might have been analyzed. However, a recent report by Poulin et al. [32], showed that chronic GVHD does not alter proliferation rates in naive or memory T cells after transplantation but reduces expression of the interleukin 7Rα chain and the antiapoptotic protein Bcl-2 on naive T cells, thus impairing survival of these cells. The increased turnover of T cells in the first 180 days after transplantation, together with the increased susceptibility of RTEs to apoptosis [33], also explains the observed diminished content of $TREC^+$ T cells in the naive T-cell compartment during this phase. When Ki67 expression declined after 180 days, TREC content in the naive T-cell compartment rapidly normalized. This demonstrates that not only thymic export of $TREC^+$ T cells, but also peripheral events, significantly influence TREC content in the naive T-cell pool in the early phase of immune reconstitution after stem cell transplantation.

We then analyzed the course of TREC content in peripheral T cells after transplantation, as shown in Figure 1, and defined 2 stages of TREC reconstitution: (1) a switch from TREC negativity (which was found in all patients analyzed) to increasingly positive TREC values at time point T_{min} and (2) a plateau level *P* of TRECs that is achieved after 18 to 24 months. These 2 stages were determined by different transplant-related factors. We found that T_{min} was strongly correlated with patient age, even in children before the onset of puberty. TRECs became measurable in a 1-year-old child after approximately 2.5 months. Each additional year delayed T_{min} by 2 weeks. Furthermore, this age dependency of T_{min} could not be alternatively explained by differences in peripheral T-cell turnover state or graft composition among the age quartiles. Certainly, this calculation underestimates the speed of thymic recovery, because—as shown previously—$TREC^+$ T cells might be short lived or might divide rapidly during this phase of immune reconstitution and remain undetectable. However, because increased T-cell division rates are comparable in naive and memory T-cell populations, we would argue that this underestimation of thymic function affects naive $CD4^+$ and naive $CD8^+$ pools harmonically. These data demonstrate that the onset of thymic output occurs much earlier than previously thought. Delineating thymic recovery from the detection of phenotypically naive T cells in the peripheral blood led to suggestions that thymic reconstitution does not start before 6 months after transplantation [34,35]. The earlier onset of thymic recovery implies that T cells with novel antigen specificities enter the peripheral T-cell pool earlier than previously thought. Vaccination programs might prove to be successful if initiated as early as 3 months after transplantation, as is currently being investigated by a study of the European Bone Marrow Transplantation Group working party for infectious diseases. A similar beneficial effect on the onset of thymic function was found for children who underwent transplantation because of a nonmalignant disease. Although the difference in T_{min} failed to reach statistical significance because of the smaller number of patients in the nonmalignant disease group, we consider this difference of almost 2 months clinically relevant. No other analyzed variables had any significant influence on T_{min}. It is interesting to note that there was no apparent difference in T_{min} and *PL* between autologous and allogeneic transplantations.

In contrast to T_{min}, *PL* was determined only by the number of transplanted $CD34^+$ stem cells and not by recipient age, disease, conditioning regimen, and so on. In this later stage of immune reconstitution, TREC levels can be regarded as a true estimate of thymic function, because the increased T-cell proliferation rate returned to baseline levels, and abnormal Bcl-2/Bax ratios on peripheral T cells, resulting in increased apoptosis, normalized after 2 years [36]. The clinical relevance of the higher thymic output in these patients is still unclear. As demonstrated previously, acute and chronic GVHD had a negative effect on thymic function, both on the onset of thymic activity and on the plateau level of thymic output, although their influence failed to reach statistical significance. This is not surprising because the thymus is a direct target of donor-antihost alloreactivity and because thymic architecture is severely disturbed in GVHD patients [37]. Whether the decline in TREC values observed after 10 years represents an artifact of the low patient number (n = 4) or is an actual phenomenon remains to be determined in a larger cohort of long-term survivors.

In summary, our data provide novel insights into the mechanisms of thymus-dependent T-cell reconstitution after stem cell transplantation in children. Most likely, the increasing age of the young patients and pretransplantation chemotherapy in children with malignant diseases will reduce thymic cellularity, thereby defining a pretransplantation level of thymic functionality that cannot be improved by any of the transplant-related factors analyzed here. This predefined status of the thymus determines the onset of thymic reconstitution after transplantation of hematopoietic progenitor cells. However, acute or chronic GVHD may result in further damage to thymic architecture. In the early phase of immune reconstitution, the rapid production of RTEs and homeostatic events contribute to refilling the peripheral T-cell pool, but these attempts are hampered by the limited availability of competent precursors and by an increased susceptibility of RTEs and mature T cells to apoptosis [32,36]. Once the T-cell dynamics have stabilized, TREC levels are higher in children who undergo transplantation with increased stem cell doses, probably as a result of a larger number of committed lymphoid precursors that are available to enter the thymic subcapsular zone.

Unfortunately, there are still no proven concepts in clinical use that clearly support thymic reconstitution. However, several promising approaches are currently being tested in animal studies, and their physiological pathways are in accordance with our observations. The use of fibroblast growth factors such as keratinocyte growth factor might restore thymic cellularity and enhance the production of thymocytes [38], whereas T cell–specific cytokines such as interleukin 7 seem to improve the survival and cycling of RTEs and peripheral T cells [39,40]. Amelioration of thymic reconstitution will certainly reconcile the skewed patterns of T-cell reconstitution after transplantation with the pathways of normal T-cell ontogeny.

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